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Mechanical stress mediated by both endosperm softening and embryo growth underlies endosperm elimination in Arabidopsis seeds.

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ABSTRACT

Seed development in angiosperms demands the tightly co-ordinated development of three genetically distinct structures. The embryo is surrounded by the endosperm, which is in turn enclosed within the maternally derived seed coat. In *Arabidopsis* final seed size is determined by early expansion of the coenocytic endosperm, which then cellularizes and subsequently undergoes developmental Programmed Cell Death, breaking down as the embryo grows. Endosperm breakdown requires the endosperm-specific basic Helix Loop Helix transcription factor ZHOUP1. However, to date the mechanism underlying the *Arabidopsis* endosperm breakdown process has not been elucidated. Here we provide evidence that ZHOUP1 does not induce the developmental Programmed Cell Death of the endosperm directly. Instead ZHOUP1 regulates the expression of cell wall modifying enzymes, thus altering the physical properties of the endosperm to condition a mechanical environment permitting the crushing of the cellularized endosperm by the developing embryo.

The “Russian doll” –like organisation of the angiosperm seed coat, endosperm and embryo implies that growth in one compartment must have direct consequences for neighbouring tissues. In *Arabidopsis* growth of the expanding coenocytic endosperm during early post-fertilization development drives seed expansion, which is in turn controlled by the seed coat in order to achieve a genetically determined final seed size and shape (Garcia et al., 2005; Garcia et al., 2003; Ingram, 2010). More recently, it has been shown that seed coat expansion is regulated by a specific cell layer, the adaxial epidermis of the outer integument, at least in part in response to mechanical tension imposed by the growing endosperm (Creff et al., 2015).

After the initial phase of rapid expansion, the endosperm cellularizes; a process which initiates in the micropylar zone surrounding the developing embryo and progresses towards the chalazal pole of the seed (Sorensen et al., 2002). Embryo growth, and a poorly characterized concurrent process involving progressive endosperm breakdown in the zone surrounding the growing embryo closely follow cellularization. In mature *Arabidopsis* seeds only a single specialized layer of cellularized endosperm

remains surrounding the mature embryo. Embryo size in *Arabidopsis* is thus strongly influenced by the size of the endosperm prior to breakdown. This relationship is dramatically altered in *zhoupi (zou)* mutants, in which endosperm breakdown does not occur. *ZOU* encodes a unique bHLH transcription factor which is expressed exclusively in the developing endosperm and acts as a heterodimer with another bHLH protein, ICE1, to mediate endosperm breakdown (Denay et al., 2014; Kondou et al., 2008; Xing et al., 2013; Yang et al., 2008). In *zou* mutants the presence of a persistent endosperm has consequences both for embryo development and for the testa. Embryo growth is dramatically reduced in *zou* mutants, due to an apparent inability of the embryo to expand into the persistent endosperm (Yang et al., 2008). The idea that the embryo is physically constrained in this background is supported by the fact that the testa in *zou* mutants is under greater tension than that in wild-type plants after endosperm cellularization (Creff et al., 2015), suggesting that the expanding embryo and the abnormally persistent endosperm compete for space within the seed cavity.

The mechanism underlying endosperm breakdown remains very poorly understood. The genetically controlled elimination of unwanted cells is a common occurrence during both animal and plant development. The term developmental Programmed Cell Death (dPCD) has been used to describe such processes (Olvera-Carrillo et al., 2015). Although in animals, the molecular control of dPCD pathways is well characterized and relatively conserved (recently reviewed in (Suzanne and Steller, 2013)), almost every reported instance of plant dPCD seems to involve unique molecular players, with the triggers of plant dPCD being particularly diverse (recently reviewed in (Van Hautegeem et al., 2014)). Interestingly however, a conserved set of indicator genes expressed prior to multiple plant dPCD processes has recently been defined (Olvera-Carrillo et al., 2015). These include the aspartate protease-encoding *PASPA3* gene, which has no known function, but is a very early marker of cells destined for dPCD, and the Bifunctional Nuclease –encoding *BFN1* gene, the expression of which has been shown to pave the way for nuclear corpse clearance during lateral root cap cell death (Fendrych et al., 2014; Van Durme and Nowack, 2016). Here we have combined biophysical and compositional analysis with expression analysis both of these pre-dPCD markers and direct *ZOU* targets, to investigate the processes involved in the breakdown of the *Arabidopsis* endosperm.

RESULTS

Cell Death markers show *ZOU*-dependent expression in the developing endosperm during seed development.

Cell death in the developing *Arabidopsis* seed is not restricted to the endosperm, and is also known to occur in the developing seed coat (Andeme Ondzighi et al., 2008; Nakaune et al., 2005) and the embryonic suspensor (Blanvillain et al., 2011). To identify cell death markers with relative specificity for the endosperm, we therefore interrogated available *in silico* resources and the literature. We identified two genes expressed prior to cell death in the lateral root cap as potentially useful pre-cell death markers in endosperm; *PASPA3* and *BFNI* (Olvera-Carrillo et al., 2015) (Fendrych et al., 2014). *In silico* expression profiles (Le et al., 2010; Winter et al., 2007) for these genes are shown in Fig S1. We characterised the expression of these genes during seed development by *in situ* hybridisation and found that their expression profiles closely matched those shown in available *in silico* and published data. *PASPA3* expression was detected in the endosperm from the late heart stage (Fig. 1A first 3 panels) while *BFNI* expression was first detected only from the late torpedo stage onwards (Figure 1A shows expression at the bent cotyledon stage). We then analysed the expression of *PASPA3* and *BFNI* in *zou-4* seeds, and found that expression of both genes was strongly reduced (Fig. 1A). This reduction was confirmed by Q-RT-PCR analysis of whole siliques from wild-type plants and homozygous *zou-4* mutants (Fig.1B, it should be noted that both *PASPA3* and *BFNI* also show expression in the testa (Fig. S1), which may explain residual expression in Q-RT-PCR experiments). The developing *Arabidopsis* endosperm thus shows *ZOU*-dependent expression of both an early and a late pre-dPCD marker.

***ZOU* activity leads to cell wall modifications in the developing endosperm.**

We previously identified a set of genes strongly down-regulated in *zou-4* mutants at the early heart stage and early torpedo stages of embryo development (Xing et al., 2013). Interestingly none of the genes identified in this analysis as strongly down-regulated (>10x) at the heart stage were annotated as being implicated in cell death, whereas several of the most strongly down-regulated genes, were annotated as potentially involved in the modification of cell wall proteins and polysaccharides. We have previously shown that one of these genes, *RGP3*, encoding a member of a small protein family shown involved in plant cell wall biosynthesis through the metabolism of Arabinose (Rautengarten et al., 2011), is a direct target of activation by the ZOU (Denay et al., 2014). We confirmed the ZOU-dependent expression of 5 more strongly regulated genes by Q-RT-PCR (Fig. 2), and confirmed their expression patterns in the developing endosperm by *in situ* hybridization (Fig. S2). Down-regulation of these genes in *zou-4* mutants could already be detected at the early heart stage of development (Fig. 2). To compare the relative spatio-temporal expression pattern of a direct ZOU target (*RGP3*) and the earliest pre-dPCD marker, *PASPA3*, we undertook an analysis using serial sections of the same seed, probed with either an *RGP3* or a *PASPA3* probe. Convincing signals were first observed for *RGP3* expression at the early heart stage, whereas *PASPA3* transcript levels were only detected at the late heart/early torpedo stages (Fig. 2).

In light of the regulation of cell-wall modifier-encoding genes by ZOU, we explored cell wall composition in the endosperm of *zou-4* mutant and wild-type seeds using antibodies against a variety of cell wall epitopes. These experiments showed that (1-5)- α -L-arabinan epitopes, labelled with the LM6 antibody (Willats et al., 1998), were more abundant in the endosperm of *zou-4* mutants than of wild-type embryos. In particular the relative staining with LM6 in the endosperm immediately adjacent to the embryo compared to that in more chalazal regions is considerably stronger in *zou* mutants compared to wild-type, and in later stages translates as a delineation of the embryo with LM6 signal, which is not observed in wild-type seeds (Fig. 2).

The endosperm of *zou* mutants is physically stiffer than that of wild-type seeds post cellularization.

In a previous study we showed that the presence of a persistent endosperm in *zou-4* mutants leads to an abnormal increase in seed size post endosperm cellularization, and causes increased tension in the developing seed coat (Creff et al., 2015). Both observations suggest that the mechanical properties of the endosperm are altered in *zou-4* mutants. To investigate this, we measured the apparent stiffness of developing seeds during development using a nano-indenter system. This equipment allows the measurement of the force necessary to carry out tissue indentations of known amplitude using a geometrically defined probe. We carried out 30µm indentations of immobilized seeds using a flat, circular probe with a diameter of 100µm as described in (Beauzamy et al., Submitted). Slopes of Force/displacement curves for the extend- and retract-phase of indentation are shown in Fig. 3 and Fig. S3 respectively (methodology described in Beauzamy et al., Submitted).

Indentations were carried out on staged seeds from wild-type and *zou-4* mutant plants from the onset of endosperm cellularization (heart stage) to bent-cotyledon stage. At the early heart stage of development we found no difference in the apparent stiffness of wild-type and *zou-4* mutant seed. However, at the early torpedo stage, when embryo elongation and endosperm breakdown is initiated in wild-type seeds, we found that the seeds of *zou-4* mutants were up to three times as stiff as those of wild-type plants at the same developmental stage. Plasmolysis of seeds significantly reduced the difference in stiffness between the two genotypes indicating that this difference can be attributed to increased endosperm turgor, coherent with previous indentation experiments (Creff et al., 2015). The lower stiffness of plasmolyzed seeds of *zou-4* mutants at the latest stage can be ascribed to arrested embryo growth in this background.

Endosperm breakdown requires embryo growth.

Our observation that the endosperm turgidity increases in the *zou-4* mutant seed, combined with the observation that the regulation of cell wall composition, rather than cell death, appears to be the primary function of ZOU, led us to ask whether endosperm death could also be dependent upon physical crushing/bursting of endosperm cells by the expanding embryo during wild-type seed development. To explore this possibility we investigated the morphology of the endosperm in mutants

in which embryo development arrests early, but where endosperm growth/cellularisation has not been described to be abnormal. In the first instance we chose two mutant backgrounds. The first homozygous for *dek1-3*, a null allele of the *DEFECTIVE KERNEL1* gene in which embryo development arrests at the globular stage, but where endosperm proliferation and cellularisation occur normally (Johnson et al., 2005; Lid et al., 2005). Because *dek1* mutants have been shown to have a partially disrupted organisation of the outer endosperm cell layer (Lid et al., 2005), we also investigated a line homozygous for the null allele of homeodomain protein-encoding gene *ATML1* (*atml1-3*), and segregating a null allele of the closely related *PDF2* gene (*pdf2-2*). Double *atml1-3 pdf2-2* mutants again arrest at the globular stage of development with no reported defects in endosperm development (San-Bento et al., 2014). For both backgrounds we fixed and resin embedded seed populations from single self-fertilized siliques at a late developmental stage when seeds containing either a *dek1-3* mutant or an *atml1-3 pdf2-2* zygotic compartment could be easily distinguished, but before seeds started to desiccate (at which point seeds with a mutant zygotic complement abort in both genotypes). In both backgrounds seeds containing phenotypically wild-type seeds, the endosperm had been eliminated. However in each case, when a seed containing an arrested embryo was sectioned, the endosperm was intact, although cells had a slightly rounded aspect as if they had separated (Fig. 4, Fig. S4).

To eliminate the possibility that *ZOU* is not expressed in mutants in which embryo development is abnormal, we analysed the expression of *ZOU*, and the direct *ZOU* target gene *RGP3* in seeds containing a *dek1-3* zygotic compartment, and phenotypically wild-type siblings, by *in situ* hybridization. We found that both genes were expressed in the endosperm of seeds containing mutant embryos. Finally, we visually compared the endosperm phenotypes of seeds containing a *dek1-3* zygotic compartment in either a *ZOU*⁺ or a *zou*⁻ mutant background. We found that the endosperm cell walls of seeds containing a *dek1-3* zygotic compartment in a *ZOU*⁺ background resembled those of wild-type seeds undergoing endosperm breakdown, in that they were relatively weakly stained with Toluidine blue and additionally showed evidence of cell-wall separation (Fig. 4). A similar phenotype is observed in *atml1-3 pdf2-2* endosperm (Fig. S4). In contrast the endosperm cell walls of seeds with

a *zou-4* or a *dek1-3 zou-4* zygotic compartment looked visually more robust, were more strongly stained, and showed no signs of cell separation.

Interestingly, we found that in siliques segregating for *dek1-3* seeds, by the bent torpedo stage of embryo development, the endosperm of seeds with a *dek1-3* zygotic compartment showed a strong, uniform expression of both *PASPA3* and *BFN1*, suggesting that the expression of these genes may be triggered in the *dek1-3* endosperm independent of the imposition of mechanical stress by the embryo (Fig. S4).

DISCUSSION

Our results strongly support the hypothesis that the loss of endosperm cells in the embryo surrounding endosperm is a process dependent upon alterations in the biochemical and physical properties of endosperm cell walls, which permit the growing embryo to expand and exert physical stresses on endosperm cells. Our analysis of pre-cell-death marker expression suggests that these physical stresses combined with embryo growth could be a trigger for a dPCD process. This does not occur in *zou* mutants due to loss of endosperm cell wall modifications. Consistent with this idea, our indentation and plasmolysis experiments, taken together with our analyses of endosperm cell wall structure and composition suggest that the cell wall modifications mediated by ZOU may affect the capacity of endosperm cells to resist an increased turgor pressure caused by endosperm compression by the growing embryo. We propose that in wild-type seeds the function of ZOU may be to render endosperm cell walls fragile enough to allow deformation, physical stress, and ultimately bursting upon compression by the growing embryo. In *zou* mutants the presence of more robust cell walls allows endosperm cells to remain intact and unstressed in the face of these compressive forces. In mutant seeds the maintenance of (1-5)- α -L-arabinan epitopes, which have previously been detected in the endosperm of *Arabidopsis* and tobacco, and shown to be most likely associated with rhamnogalacturonan-1 pectins in these tissues (Willats et al., 2001; Lee et al., 2012; Lee et al., 2013), suggests that ZOU could act to remove or modify pectins in endosperm cell walls.

Our analysis of the *dek1-3* mutant indicates that the expression of *ZOU* and *ZOU*-dependent genes in the endosperm is not dependent upon embryo growth. In addition *ZOU*-dependent endosperm cell wall thinning appears to occur normally in seeds containing a *dek1-3* zygotic compartment. *ZOU* dependent endosperm modifications are thus likely to be an autonomous feature of endosperm development, although an influence from arrested embryos cannot formally be excluded. Intriguingly, we show that the expression of known pre-cell-death markers in the endosperm, which is absent in the *zou-4* mutant, is not lost in seeds containing a *dek1-3* zygotic compartment. *ZOU*-mediated cell wall modifications may therefore impose a physiological stress upon endosperm cells, which ultimately leads to the expression of these markers even in the absence of embryo-mediated compression. The timing of this expression in comparison to that in wild-type siblings was difficult to ascertain and it could therefore be an early consequence of the onset of abortion. However, the fact that the cells in *dek1-3* endosperms are capable of active gene expression indicates that they are still alive at the late-torpedo stage, when much of the endosperm in sibling seeds has been eliminated, underlining the requirement for physical stresses imposed by the embryo in cell elimination.

Cell wall weakening and subsequent cell rupture due to the behaviour of neighbouring cells may be a common feature involved in sensitizing or conditioning plant cells for dPCD during cell elimination. For example the death in the lateral root cap (lrc) cells is preceded by cell extension driven by the expansion of underlying tissues (Fendrych et al., 2014). Sensitivity to this extension is conditioned by expression of the SOMBRERO transcription factor (Fendrych et al., 2014), in a mechanism that could be functionally similar to that mediated by *ZOU*. More detailed biophysical analysis as well as detailed cell structure analysis will help to clarify whether molecular pathways are shared between these processes and with other dPCD events in plants, but may be hampered by the relative inaccessibility of the developing endosperm.

MATERIALS AND METHODS

Plant material

All genotypes used in this work have been previously described and published. Seeds were plated on Murashige and Skoog (MS) media, vernalized for 3 days at 4°C, germinated under short day conditions (8 hours light) at 18°C. Plantlets were transferred to soil in identical growth room conditions for 3 weeks and finally placed under continuous light at 16°C. To ensure synchronicity between plants, flowers were labelled and dated at anthesis.

Resin embedding an immunolocalisation experiments

Embedding and immunolocalisations were carried out as described in (Creff et al., 2015). Toluidine blue staining was carried out as described in (Denay et al., 2014).

In situ hybridisation experiments

In situ hybridisation experiments were carried out as described in (Creff et al., 2015). Primers used to amplify probe fragments from seed cDNA are shown in supplementary Table 1.

RNA extraction and Q-RT-PCR experiments

RNA extraction and Q-RT-PCR experiments were carried out as described in (Creff et al., 2015). Primers used in Q-RT-PCR amplifications are shown in supplementary Table 1.

Nano indentation of developing seeds and analysis of indentation curves

Indentation experiments and statistical analysis were carried out using a TI 950 TriboIndenter (Hysitron) exactly as described in (Beauzamy et al., Submitted). Slopes were taken from the region of the extend force-displacement curve ranging from 75 to 100% of the maximum force.

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FIGURE LEGENDS

Figure 1 - Expression of the cell death marker-genes in the developing endosperm is dependent upon the activity of ZOU. A) Detection of *PASPA3* and *BFN1* transcripts by *in situ* hybridisation in wild-type and *zou-4* seeds. Signal is shown as blue/black colouration (white arrows). Red/brown staining of the endothelium is a background artefact in seeds. Scale bar = 50 µm. B) Q-RT-PCR analysis of *PASPA3* and *BFN1* transcripts relative to *EIF4* transcripts in whole siliques from wild-type and *zou-4* plants. Error bars are standard deviations from three biological replicates.

Figure 2 - ZOU activity is required for cell wall modification in the developing endosperm.

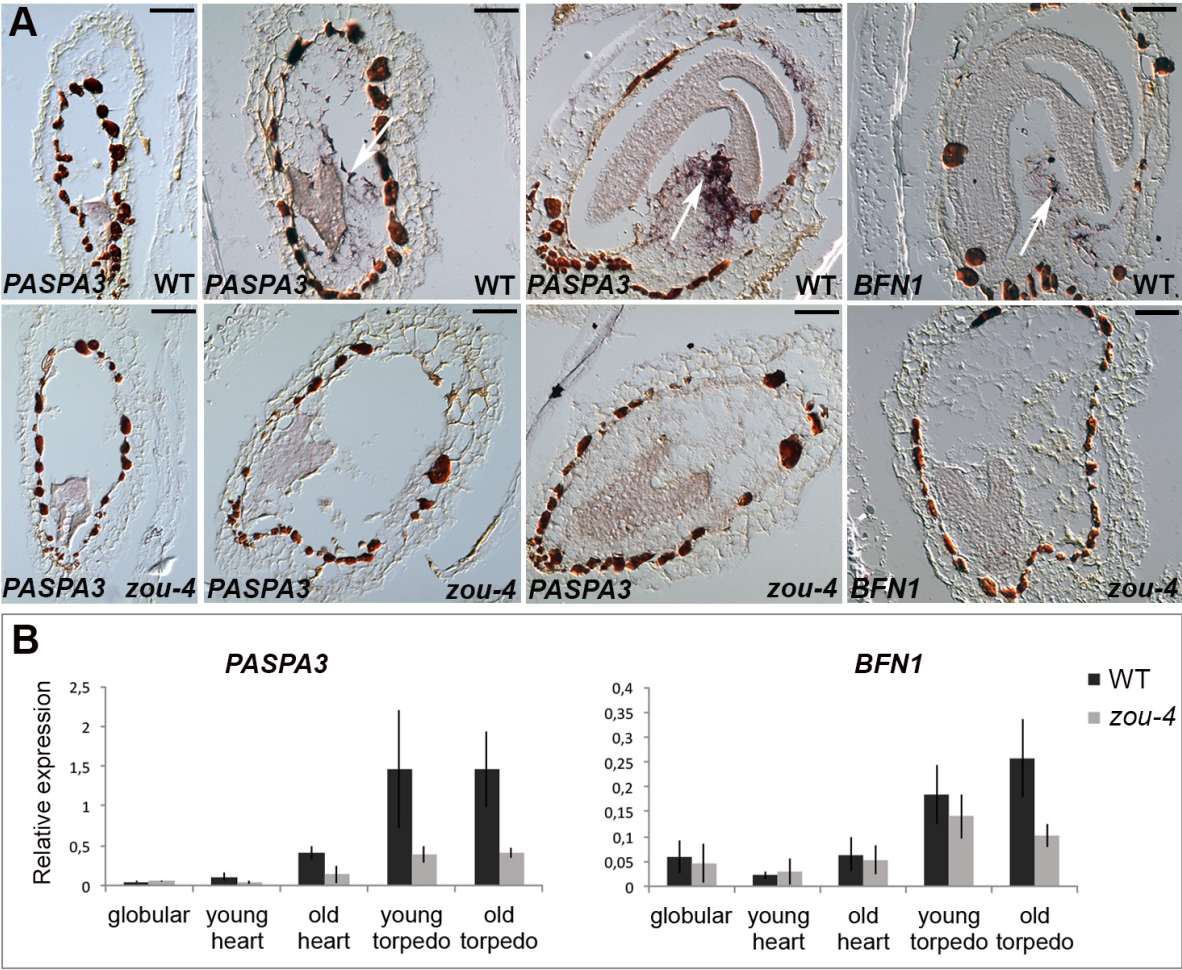
A) Q-RT-PCR analysis of transcript accumulation from ZOU-dependent genes annotated as

encoding cell wall modifiers, relative to *EIF4* during wild-type and *zou-4* seed development. Error bars are standard deviations from three biological replicates. B) Analysis of *RGP3* and *PASPA3* transcript accumulation by *in situ* hybridisation. Upper and corresponding lower panels show serial sections from the same seed. Signal is shown as blue/black colouration (white arrows). Red/brown staining of the endothelium is a background artefact in seeds. C) Immunolocalisations using the LM6 antibody in wild-type and *zou-4* mutant seeds during development. White arrows indicate intense signal at the embryo surface in *zou-4* mutant seeds. Scale bar = 50 μ m.

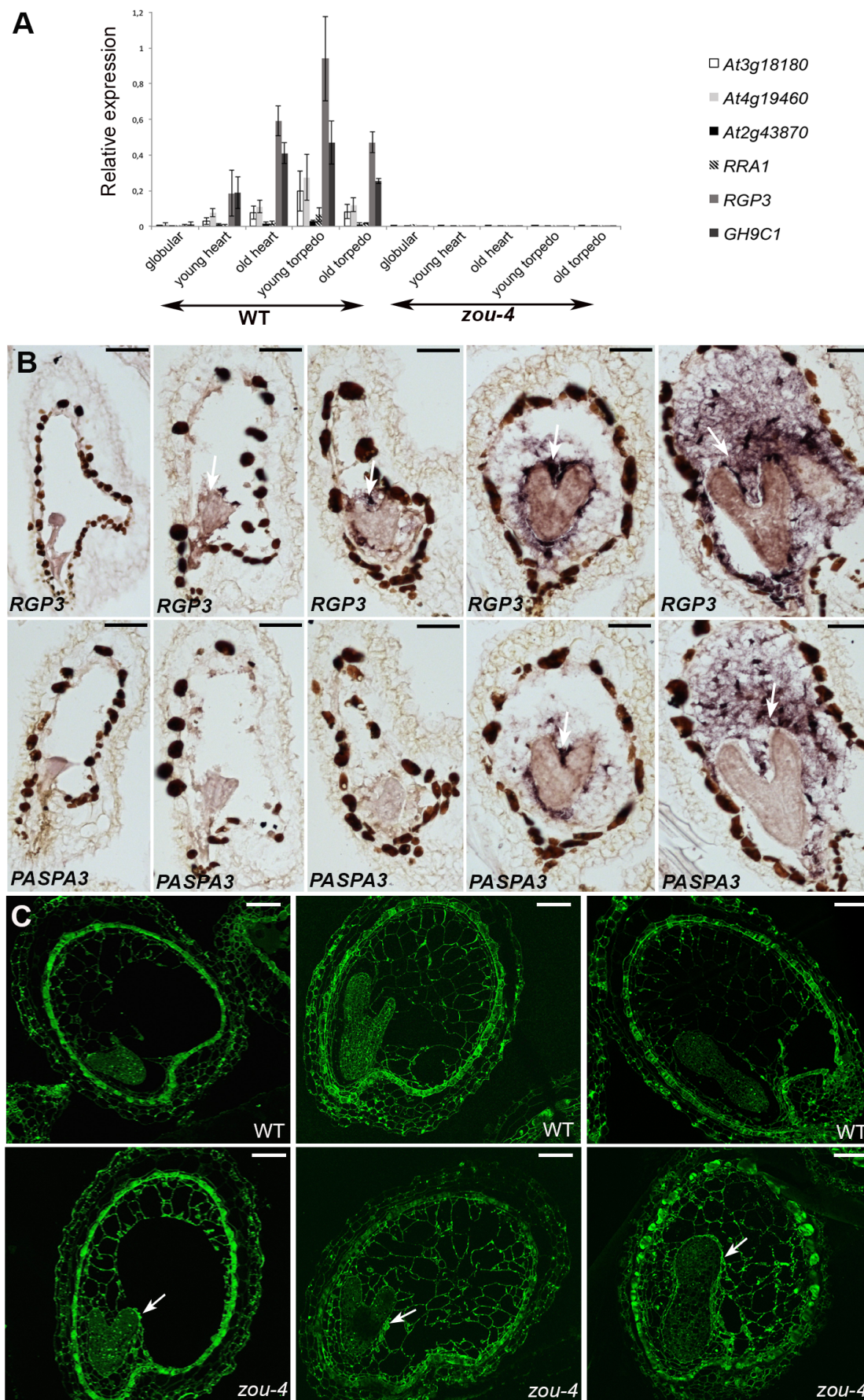
Figure 3 - ZOU activity controls seed turgor during embryo expansion. Comparison of the apparent stiffness of wild-type and *zou-4* mutant seeds at different developmental stages. Stiffness values were extracted from the late linear extend phase of force/displacement curves obtained using a nano-indenter as described in (Beauzamy et al., Submitted). Effects of osmotic treatment (0.7M mannitol for 90 minutes) on seed stiffness are shown. Developmental stages of wild-type seeds are shown. Differences between populations were evaluated statistically using a Wilcoxon rank-sum test. ***= $p < 0.001$. Error bars indicate standard deviation around the arithmetic mean.

Figure 4 - Embryo growth is required for endosperm breakdown but not cell wall modification. A) Toluidine-blue stained resin sections showing the structure of wild-type, *dek1-3*, *zou-4* and *dek1-3 zou-4* endosperm at the same developmental stage. Arrows indicate the position of the embryo. B) Analysis of *ZOU* and *RGP3* transcript accumulation by *in situ* hybridisation in wild-type and *dek1-3* seeds. Signal is shown as blue/black colouration (arrows). Red/brown staining of the endothelium is a background artefact in seeds. Scale bar = 50 μ m.

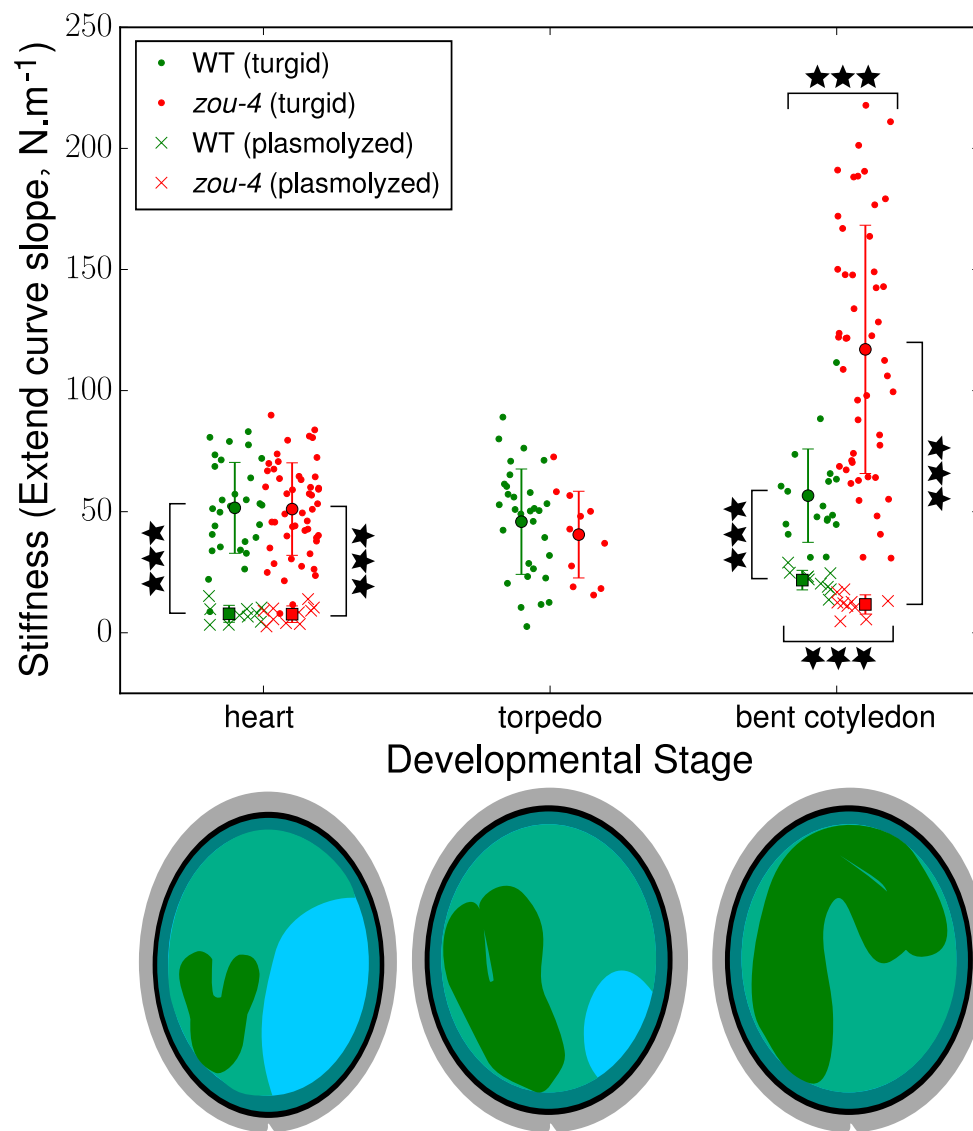
Fourquin et al Fig. 1



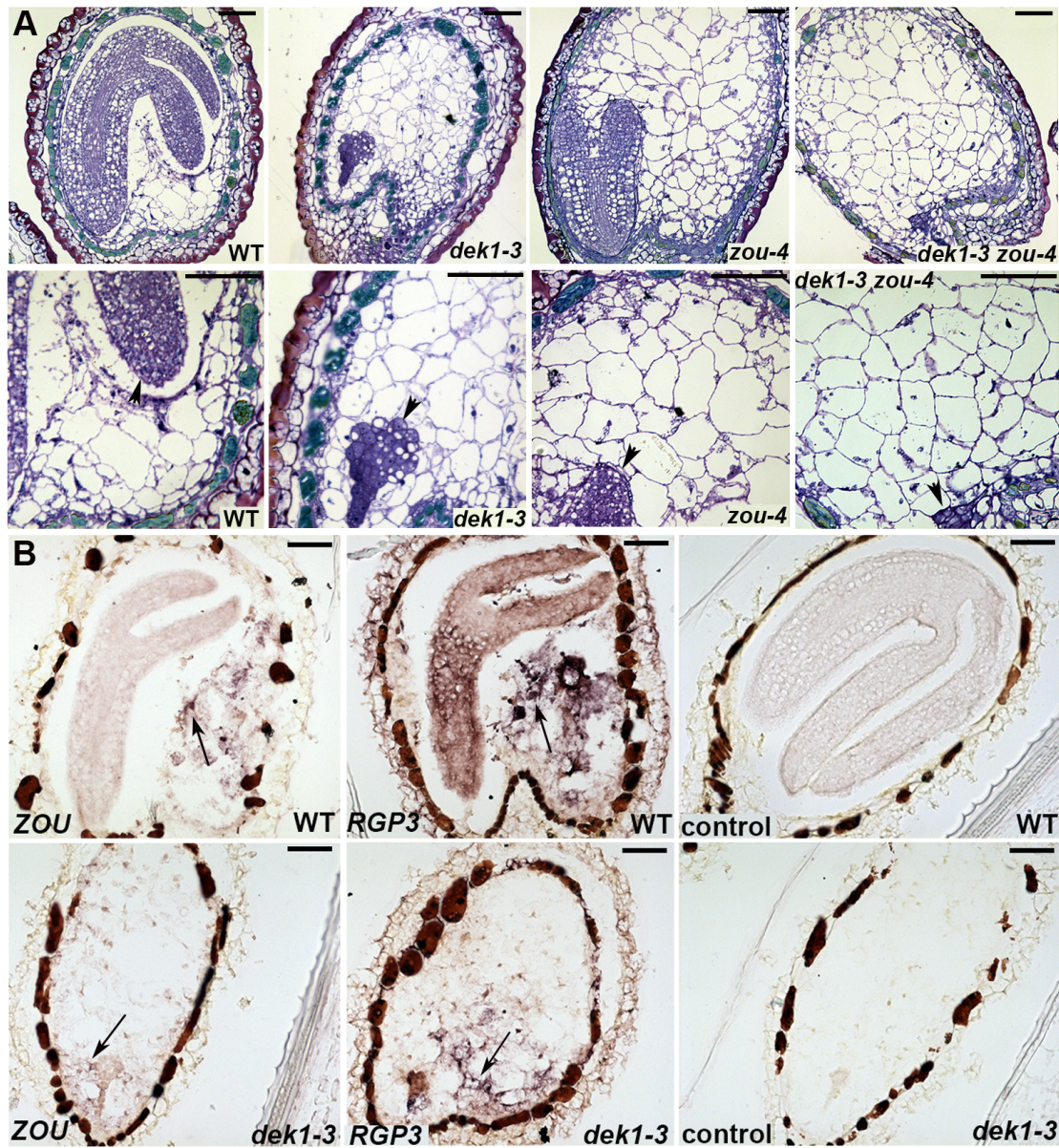
Fourquin et al Fig. 2



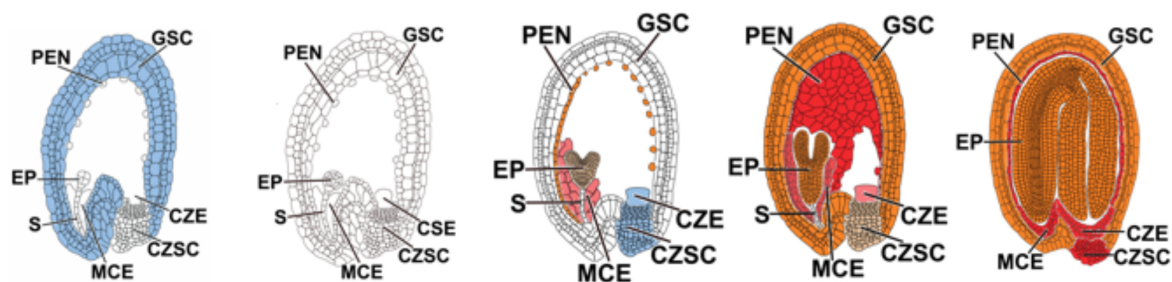
Fourquin et al Fig. 3



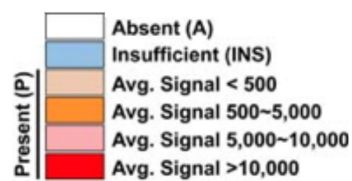
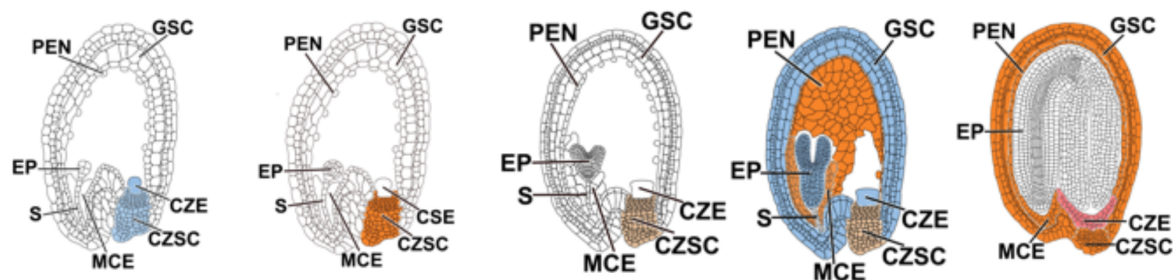
Fourquin et al Fig. 4



PASPA3

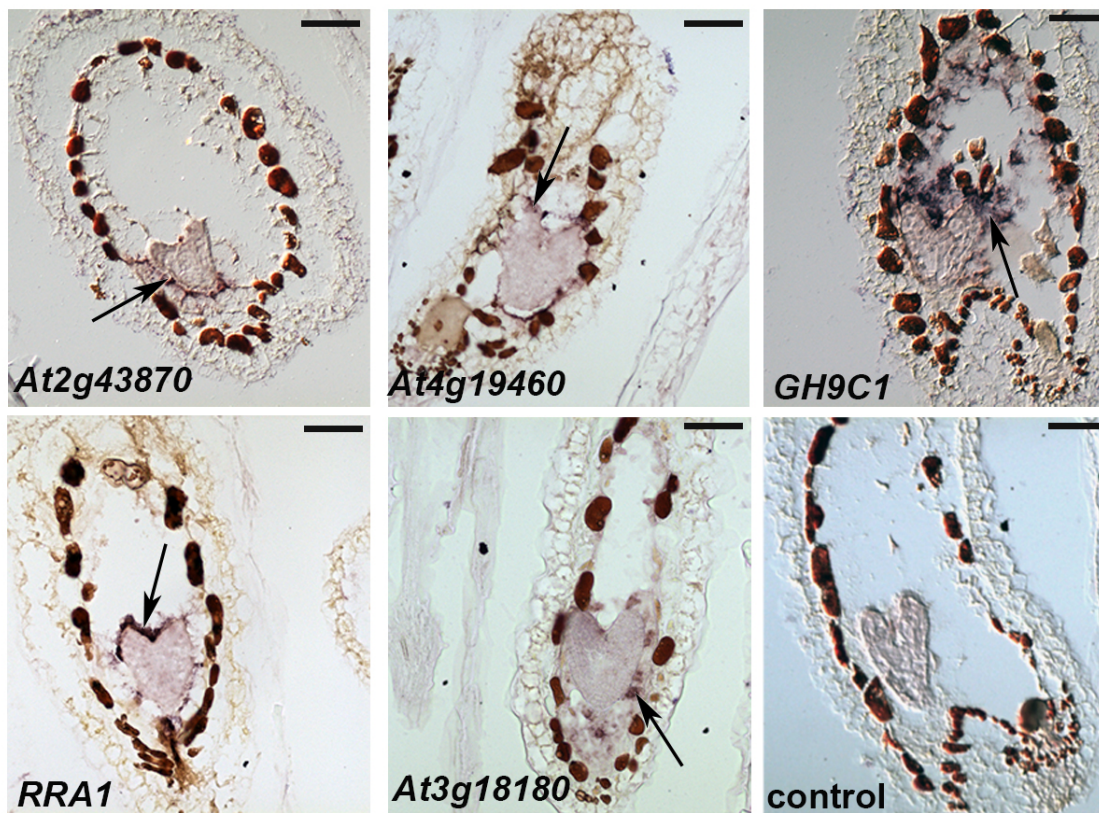


BFN1



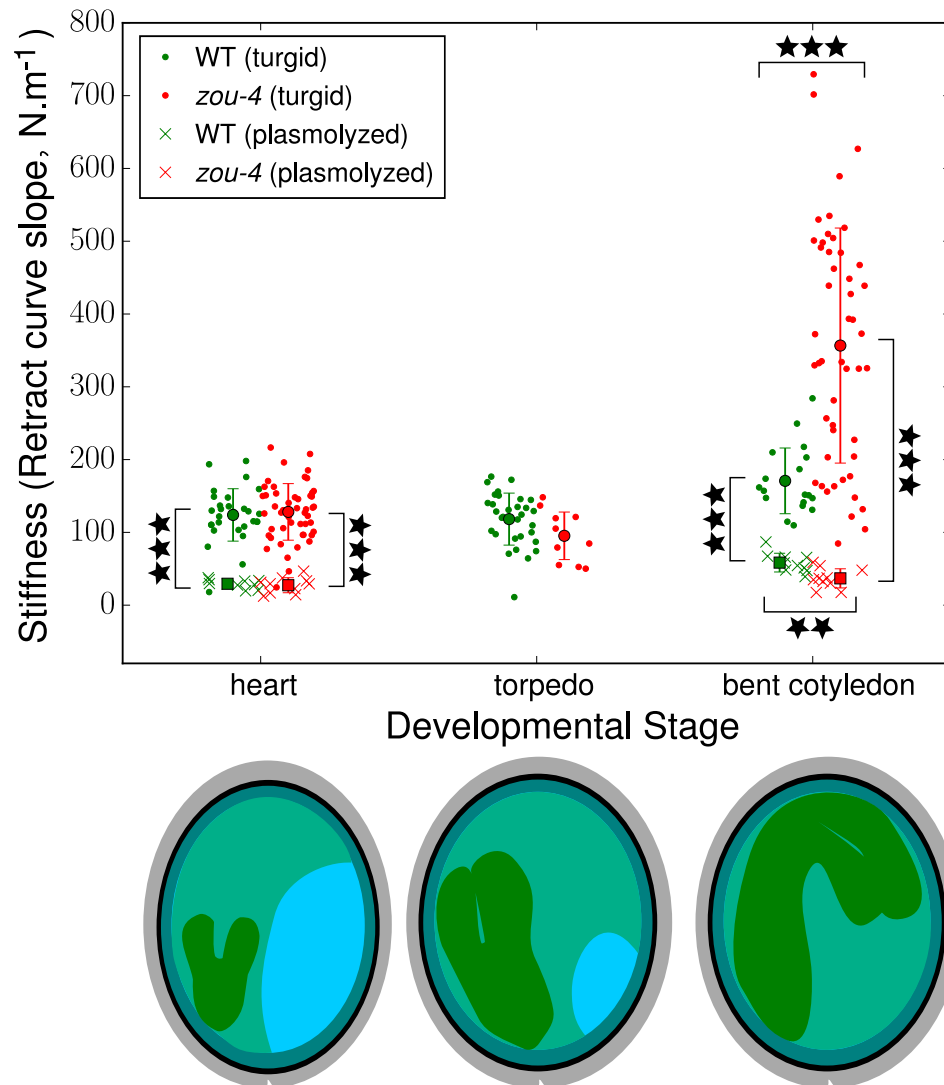
Supplementary Figure 1

Expression data for *PASPA3* and *BFN1* downloaded from the Seed Gene Network resource (<http://seedgenenetwork.net/>)



Supplementary Figure 2

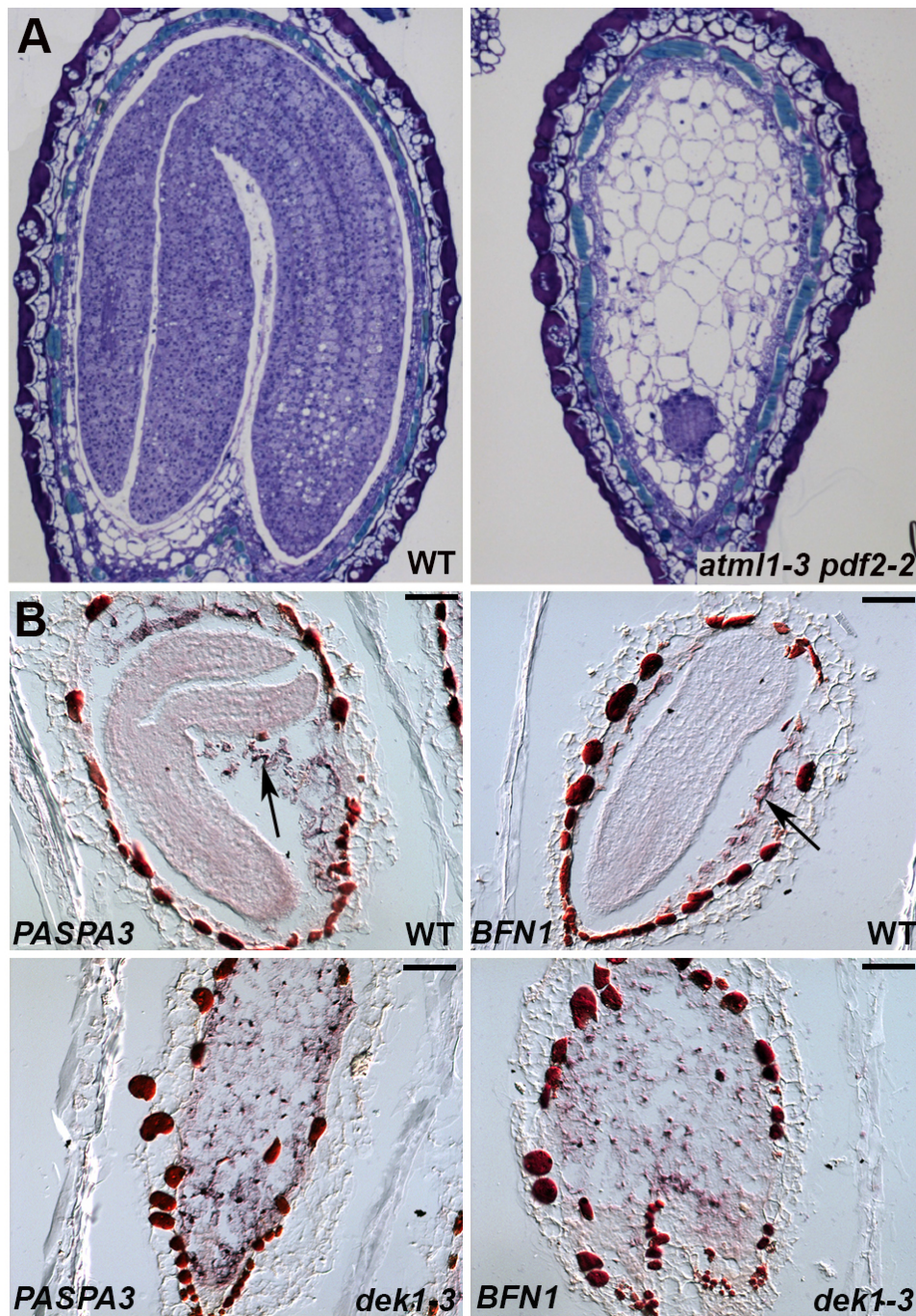
Analysis of transcript accumulation from ZOU-dependent genes annotated as encoding cell wall modifiers by *in situ* hybridisation in wild-type seeds. Signal is shown as blue/black colouration (white arrows). Red/brown staining of the endothelium is a background artefact in seeds. Scale bar = 50 μ m.



Supplementary Figure 3

Comparison of the apparent stiffness of wild-type and *zou-4* mutant seeds at different developmental stages. Stiffness values were extracted from the early linear retract phase of force/displacement curves obtained using a nano-indenter as described in (Beauzamy et al., Submitted). Effects of osmotic treatment (0.7M mannitol for 90 minutes) on seed stiffness are shown. Developmental stages of wild-type seeds are shown. Differences between populations were evaluated statistically using a Wilcoxon rank-sum test. **= $p < 0.01$, ***= $p < 0.001$. Error bars indicate standard deviation around the arithmetic mean.

Fourquin et al Fig. S4



Supplementary Figure 4

A) Toluidine-blue stained resin sections showing the structure of wild-type, and *atml1-3 pdf2-2* endosperm. B) Analysis of *PASPA3* and *BNF1* transcript accumulation by *in situ* hybridisation in wild-type and *dek1-3* seeds. Signal is shown as blue/black colouration (white arrows). Red/brown staining of the endothelium is a background artefact in seeds. Scale bar = 50 µm.

Fourquin et al Supplementary Table S1

Primers Name	Primers Sequence
	AMPLIFICATION OF IN SITU PROBES
At3g18180attB1ATG	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAACAATGACAAAGAAGGAT ATTC
At3g18180attB2STOP	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACACTGACTGATTATGC AATAG
At4g19460attB1ATG	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAACAATGAATCCTTTAATT GAAC
At4g19460attB2STOP	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAAGGGTAAATACAAAAT TTCTG
At1g75120attB1ATG	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAACAATGGCGGTTTCGTAA AGAGAAAG
At1g75120attB2STOP	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATGAACCATCACGGAA C
At2g43870attB1ATG	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAACAATGGCTTCACTTCTT GTCCTC
At2g43870attB2STOP	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATAGACAATTTGGCTG
GH9C1attB1ATG	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAACAATGAGGAAGTTTGGT GGATC
GH9C1attB2STOP	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGTTGTAACCTCAAACT GAG

	Q-RT-PCR ANALYSIS
PASPA3 F	CTCTGTAAAAATGGGAAGTAGGTTC
PASPA3 R	CTCTCCTTTGAAGTGCTTCGG
BFN1 F	CATCGGCTTTTAGATCATCCAC
BFN1 R	CACTTCTAAACAAAGCAGTCCAC
q18180For	CTCAGCCCTTTACCAATCGT
q18180Rev	TTCTTTGGAGATTGTAGTGTTGGT
q19460For	AAGCTTTGACGGCGGTTAT
q19460Rev	TCTCTCCGCCAATCTCTCC
qRRA1For	TCAGATAAACTCGAAAGAATGCAA
qRRA1Rev	CATCTTGCTTGCCATTAACGTA
q43870For	AGCTTCTTTATGGGATTGTAAAAAGT
q43870Rev	TGAGCTCTGAAATCCTATTGTCG
qGH9C1F	GGCCTATTCGCTAAACTCTATGGAG
qGH9C1R	ATTTGTGCACCTGATTGCTTG
qRGP3L	ACACCATTGATGATGATTGCTT
qRGP3R	TGTTAAAGAAATGCGGAGTTGA

